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TITLE: A Unique Class of Topoisomerase Mutants that are Hypersensitive to Multiple Antitumor Agents

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The goal of this research is to understand the detailed mechanism of action of antitumor drugs that target type II topoisomerases. Previous analysis showed that a drug resistant bacteriophage T4 mutant harbored two amino acid substitutions (S79F, G269V) in topoisomerase subunit gp52. When both mutations are present, the G269V substitution suppresses a topoisomerase negative phenotype caused by the S79F substitution while the G269V substitution by itself was shown to confer hypersensitivity *in vivo* (*Cancer Research* **58**, 1260-1267). In order to understand these phenotypes on a biochemical level, I purified the S79F and G269V single mutant enzymes as well as the S79F/G269V double mutant enzyme. I found the G269V enzyme to be hypersensitive to a number of cleavage-inducing antitumor agents and it displayed an apparent 10-fold increase in drug-independent DNA cleavage, suggesting a novel mechanism of sensitivity in which the enzyme equilibrium has been shifted to favor the cleavage complex. The S79F single and S79F/G269V double mutant enzymes were resistant to the tested drugs. This resistance is likely due to an altered drug-binding pocket created by the S79F substitution. Unexpectedly, the S79F mutant enzyme has a defect in ATP dependence that is suppressed by the G269V substitution.

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Introduction

The goal of this research is to understand the detailed mechanism of action of antitumor agents that target type II topoisomerases. This includes some of the most widely used chemotherapy drugs: doxorubicin (adriamycin), mitoxantrone, and the epipodophyllotoxins VP-16 (etoposide) and VM-26 (tenoposide) (3). Two of these drugs, doxorubicin and mitoxantrone, are commonly used in the treatment of breast cancer patients (3). The resistance of certain cancer cells, including some breast cancer cells, to these drugs is still a major problem in chemotherapy. One of the ways cancer cells can acquire resistance to these topoisomerase II inhibitors is by producing an altered form of the enzyme that is no longer sensitive to the drugs (1). Detailed studies of a number of these resistant forms of topoisomerase II have contributed greatly to our understanding of drug action. We report here the biochemical analyses of two T4 topoisomerase mutants that display unique properties on their own and interact with one another in an interesting way. These studies have shed new light on the mechanism of action of type II topoisomerases and their interactions with inhibitors.

Body

Background. My original proposal describes in detail the genetic isolation of the G269V mutant topoisomerase strain (see ref 9, p. 8). Briefly, a T4 gene *52* drug-resistant mutant was sequenced and was found to harbor two amino acid substitutions: S79F and G269V (2). When both substitutions are present, the G269V substitution is thought to suppress a topoisomerase negative phenotype caused by the S79F substitution alone. However, when the G269V mutation was substituted into a wild-type background, the resulting phage exhibited hypersensitivity to *m*-AMSA and oxolinic acid *in vivo* (2). This result was unexpected as the G269V substitution is located in a domain of the topoisomerase that was not thought to play a role in drug sensitivity, namely, the tower domain (see ref 10 and references therein for a discussion of these domains). With one possible exception (a complex triple mutant of yeast topoisomerase II, 6), this is the only mutation in the tower domain of any type II topoisomerase known to affect drug sensitivity.

We feel that the G269V mutant enzyme defines a new class of mutants that are hypersensitive to all drugs. However, it is still unclear how a substitution in the tower domain leads to suppression of the S79F substitution located in the CAP-like domain. The analyses of these two mutants and the isolation of additional tower domain mutants with altered drug sensitivities could provide us with new insights into the mechanism of antitumor drug action.

Detailed summary of data. We have completed the biochemical analysis of the G269V mutant enzyme (Task 1) and have published our findings in *Biochemistry* (10, see also Appendix II). Briefly, the G269V mutant appears to be very different than any previously identified topoisomerase mutant. For one thing, the substitution is located in a region of the protein that has not been previously implicated in drug or DNA binding. Further, unlike other mutants, the G269V enzyme is hypersensitive to a broad range of

topoisomerase inhibitors but does not have an altered drug binding pocket. We believe that the G269V substitution causes hypersensitivity by increasing the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. This is the first type II topoisomerase mutant described that alters drug sensitivity by altering the equilibrium of the enzyme.

Recall that the G269V substitution was originally isolated in combination with S79F in a selection for drug-resistant mutants (2, 4). S79F by itself causes a topoisomerase negative phenotype *in vivo*, which is rescued by the G269V substitution. Comparisons with the yeast enzyme place the S79 residue in the CAP-like domain of the protein, far from the G269V substitution in the tower domain (these domains are detailed in ref 10; for a picture Figure 6A of Appendix II). It is unclear how a substitution in the tower domain leads to suppression of a substitution in the CAP-like domain. What is the nature of the defect of the S79F mutant and how does G269V suppress this defect? Also, why is the drug-resistance phenotype dominant to the hypersensitivity phenotype? We feel that the answers to these questions could lead to a further understanding of the hypersensitivity phenotype (Task 2).

In order to determine the nature of the defect in the S79F containing phage strain, I purified the S79F mutant enzyme by utilizing the T4 topoisomerase overproduction strain (see proposal). My preliminary results with the purified S79F mutant enzyme were detailed in my last annual report and will be reviewed briefly here. Unexpectedly, the purified S79F protein was proficient at relaxing supercoiled DNA with a specific activity similar to that of the wild type enzyme (1.7×10^6 U/mg and 2.9×10^6 U/mg, respectively). Thus, the S79F mutant enzyme is apparently proficient for DNA binding, DNA cleavage, strand passage and religation.

We next analyzed the drug sensitivity spectrum of the S79F mutant enzyme. Since the G269V substitution causes hypersensitivity to all of the drugs, it is reasonable to assume that the S79F substitution is responsible for conferring the drug-resistance phenotype displayed by the double mutant enzyme. Therefore, we would expect the S79F single and S79F/G269V double mutant enzymes to have the same drug-sensitivity spectra. Indeed we found this to be the case with the S79F substitution conferring resistant to *m*-AMSA, ellipticine, 2-me-9OH E^+ and oxolinic acid and partial sensitivity to VP-16, VM-26 and mitoxantrone (O'Reilly and Kreuzer, unpublished data, 5).

In the relaxation and cleavage assays described above the S79F mutant enzyme appears to be proficient at many steps of topoisomerase reaction pathway. However, these experiments were performed with supercoiled circular plasmid DNA and bacteriophage T4 DNA is modified with glucosylated hydroxymethyldeoxycytosines in place of deoxycytosines. Therefore, cleavage assays were performed with the S79F mutant and wild-type enzymes in the presence of purified modified T4 DNA and VP-16. We found that the S79F mutant enzyme was also proficient in binding and cleaving T4 modified DNA (data not shown).

Thus, under the conditions tested, the catalytic defect of the S79F mutant enzyme was not obvious. There are a number of possible explanations for this apparent difference between the *in vivo* and *in vitro* results. Certainly the mutant enzyme could simply be unstable *in vivo*. However, this is unlikely as the purification resulted in protein yields similar to those obtained from other mutant enzyme preparations. Alternatively, the mutant could be defective in something specific like the decatenation of replicated DNA. Further, the mutant may have specific requirements for salt, ATP, magnesium or other cofactor and our buffers may be more permissive than the *in vivo* environment of the cell. Further, the topoisomerase negative phenotype was more dramatic in some strains of bacteria than others (2). This observation leads to the intriguing possibility that the mutant protein may be defective in some essential protein-protein interaction with a specific host factor.

During the review process of our manuscript on the G269V mutant enzyme we came upon testing the ATP dependence of our enzymes. Prior work with human topoisomerase II mutant enzymes has shown a correlation between ATPase function and drug sensitivity (8). One of the reviewers of our paper wondered if we might see a similar coupling between ATPase activity, DNA cleavage levels and drug sensitivity. However, we found the G269V mutant enzyme to be equally hypersensitivite to *m*-AMSA and oxolinic acid in both the absence and presence of ATP (data not shown). Further, we found that DNA cleavage by the wild-type enzyme was ATP independent (see also 7).

Although drug sensitivity was not affected by the absence or presence of ATP we did notice something interesting in the reactions lacking ATP. The G269V mutant enzyme was much more effective than the wild-type at relaxing DNA in the absence of ATP (Figure 1). Most type II DNA topoisomerases require ATP in order to relax DNA in a processive manner (11). However, the T4 type II topoisomerase is known to exhibit a low level of DNA transport activity in the absence of ATP (11). Thus, the wild-type enzyme is able to relax the DNA to some extent in the absence of ATP (Fig 1 lane 4; enzyme dimer-to DNA ratio of 1). On the other hand, the G269V mutant enzyme was much more capable of relaxing the DNA in the absence of ATP, (Figure 1 compare lane 4 to 5). We wondered if this ability to relax DNA in the absence of ATP was somehow related to the suppression of the S79F catalytic defect.

To address this possibility, we performed a time-course experiment and compared the relaxation abilities of our four enzymes in the absence of ATP (the wild-type enzyme, the G269V and S79F single mutant enzymes, and the S79F/G269V double mutant enzyme). As shown in Figure 2, the wild-type enzyme is able to relax the DNA over time however; the DNA never becomes fully relaxed (lanes 2-6). On the other hand, the G269V mutant enzyme is able to fully relax the DNA by the 75 min time point (Figure 2, lane 9). Both of these results are in sharp contrast to those of the S79F single mutant that has not relaxed any DNA even after 22 hours (Figure 2; lanes 12-16). Interestingly, the S79F/G269V double mutant displays an intermediate ability to relax DNA in the absence of ATP (Figure 2; lanes 17-21). It should be noted that all of the enzyme are active throughout the duration of the experiment as all four proteins are fully capable of relaxing the DNA upon the addition of ATP (0.5 mM) after the last time-point (data not shown).

These results support the hypothesis that the S79F substitution causes a defect in ATP dependence that is suppressed by the G269V substitution. This defect could be in ATP binding, hydrolysis or even the coupling of these activities with catalysis.

Although the above results seem convincing there are a number of caveats associated with them. First, it is possible that small amounts of ATP have co-purified with our enzymes. This amount is probably small and we assume uniform between preps as they were performed in an identical fashion. Nonetheless, it is possible that even very small differences could be affecting our results. Further, the above experiments were performed at enzyme dimer-to-DNA ratios of 1 (35 fmol of both DNA and topoisomerase dimer) and it would be reassuring to see this effect with catalytic amounts of protein. Finally, although all of our enzymes have similar specific activities, they are not identical and we need to make sure we are seeing specific differences in ATP dependence and not general enzyme activities.

To address these points we performed relaxation assays in the presence of varying ATP and protein concentrations (Figure 3). At 0uM ATP, we see that the G269V single mutant enzyme is more ATP independent than the wild-type enzyme even at enzyme dimer-to-DNA ratios of 0.1 (Figure 3a, compare lanes 2-4 with 5-7). Further, the defect caused by the S79F substitution is again partially rescued by the G269V substitution (Figure 3a, compare lane 10 to 13). The next concentration of ATP, 10 μM, is 50 times less than we use in our standard reactions but still in vast excess to our enzyme and substrate. At this level the apparent ATP independence of the G269V mutant enzyme is less evident compared to wild-type especially at the highest enzyme dimer-to-DNA ratio of 1 (Figure 3b, compare lanes 2-4 with 5-7). However, the rescue of the S79F substitution by the G269V substitution is still clearly visible (Figure 3b, compare lanes 8-10 with 11-13). At 500 μM ATP the DNA is fully relaxed in most of the reactions however we can still see the rescue of the S79F substitution by the G269V substitution at the lowest enzyme dimer-to DNA ratio, 0.01 (Figure 3c, compare lane 8 to 11).

Thus, the defect of the S79F substitution and its rescue by the G269V substitution are observed at three different enzyme dimer-to-DNA ratios as well as three different ATP concentrations. If the effects at 0 μM ATP were due to contaminating ATP from our preps we would likely not observe the "defect and rescue" at the higher ATP levels. These results also suggest that we are not being misled by subtle differences in the specific activity of our enzymes. For example, the biggest difference in specific activity is less than 2-fold between the wild-type enzyme (2.9×10^6 U/mg) and the S79F single mutant enzyme (1.7×10^6 U/mg). Yet, using ten times more of the S79F mutant enzyme did not cause 5-fold more relaxation as would be predicted (Figure 3b, compare lane 3 with 10). Taken together, these results show that the S79F single mutant has a specific defect in ATP dependence that is suppressed by the G269V substitution.

We next performed an ATP titration to determine the concentration required by each enzyme to fully relax the DNA. The wild-type, G269V single mutant, and S79F/G269V double mutant enzymes were all able to fully relax the DNA at 50 μM ATP while the S79F single mutant required 200 μM ATP (Figure 4, enzyme dimer-to-DNA ratio 0.1).

Although these titrations need to be expanded for accurate quantitation, the S79F mutant enzyme requires at least a four fold higher concentration of ATP than the wild-type enzyme. These results strongly suggest that S79F mutant enzyme has a higher K_m for binding ATP and thus, the defect is likely to be associated with ATP binding.

At this point it is unclear if this observed defect in ATP dependence is related to the topoisomerase negative phenotype observed in infected cells. Estimates of the intracellular concentration of ATP are in the mM range, well above the 200 μM required for the S79F enzyme. However, it is likely that concentration of ATP is not uniform throughout the cell. Further, many cellular enzymes utilize ATP and it is possible that the S79F enzyme cannot compete with them effectively. With this in mind, we would like to know how the S79F mutant behaves *in vivo*. We plan to perform DNA relaxation and replication assays during infections of *E. coli* containing a suppressing Phe-tRNA with the $S79^{am}$ and wild-type phages. Will the mutant be proficient for both activities albeit slow? What if the mutant is proficient for DNA relaxation but deficient for DNA replication? Can we suppress any defects by supplementing the media with ATP or even glucose?

Even if we cannot firmly establish a direct relationship between the ATP defect of S79F and the *in vivo* phenotype this finding still has broad implications for the structure and function of type II topoisomerases. Comparisons with the yeast and bacterial enzymes place the S79 residue in the CAP-like domain of the protein and it is somewhat surprising to find a mutant with an ATP defect in this region. While many drug resistant mutations from numerous systems have been shown to reside in the CAP-like domain, none of them have been demonstrated to affect ATPase function. Instead, these mutants are thought to act by altering the drug-binding pocket. The S79F substitution likely causes drug resistance in this manner because we have already shown that the G269V substitution does not alter the drug-binding pocket while that of the doubly substituted enzyme is altered (5, 10). It is unclear if the ATP defect of the S79F substitution is related and/or enhances drug resistance levels. Nonetheless, this is the first demonstration of a mutation in the cleavage-religation portion of the protein that affects ATP function.

Key Research Accomplishments (Items in bold are new for this year)

Task 1 (Also detailed in O'Reilly and Kreuzer, 2002, included as Appendix II)

- Purified the G269V mutant topoisomerase protein from *E. coli* cells that were infected with bacteriophage T4.
  - The G269V protein behaved the same as the wild-type enzyme during all steps of the purification process.
- Determined that the specific activity of the G269V mutant enzyme was similar to that of the wild-type enzyme (2.0×10^6 U/mg and 2.9×10^6 U/mg respectively).
- Demonstrated that the G269V mutant enzyme is detected in cleavage complexes at roughly ten times the level of the wild-type enzyme.

- Determined the drug-sensitivity spectrum of the G269V mutant enzyme (qualitative).
    - The G269V mutant displayed hypersensitivity to all of the drugs tested.
- Found that the mutant enzyme does not seem to have an altered DNA sequence specificity compared to that of the wild type enzyme.
    - This suggests a novel mechanism for altered drug sensitivity.
- Modified a published filter-binding assay for use in the quantitation of topoisomerase cleavage complexes.
- Quantitated the levels of drug sensitivity of the G269V mutant enzyme compared to the wild-type enzyme.
- **Demonstrated that the G269V mutant enzyme is equally hypersensitive to *m*-AMSA and oxolinic acid in both the absence and presence of ATP.**
- **Demonstrated that DNA cleavage by wild-type T4 enzyme and G269V mutant enzyme is ATP independent (see also Kreuzer and Alberts, 1984).**

Task 2 (unpublished results)

- Purified the S79F mutant enzyme from infected *E. coli* cells (also did a purification of the S79F/G269V double mutant enzyme).
    - The S79F and S79F/G269V proteins behaved the same as the wild-type enzyme during all steps of the purification.
- Determined the specific activities of the mutant enzymes. Both similar to that of the wild-type enzyme (S79F, 1.7 X 10^6 U/mg; S79F/G269V, 2.2 X 10^6 U/mg; wild-type, 2.9 X 10^6 U/mg).
- Determined the drug sensitivity spectrum of the S79F mutant enzyme in order to compare it to the previously characterized S79F/G269V double mutant enzyme (qualitative).
    - The S79F single and S79F/G269V double mutant enzymes share similar drug sensitivity spectra.
- Found that the S79F mutant enzyme can recognize and cleave T4 modified DNA.
- **Found the S79F mutant enzyme to have a defective in ATP dependence that is suppressed by the G269V substitution.**
- **Determined that the S79F mutant enzyme requires at least four times more ATP than the wild-type enzyme to achieve full relaxation.**
- **Showed this ATP deficiency to be apparent even when catalytic amounts of enzyme were used.**

Task 3 (unpublished results, detailed separately in Appendix I)

- **Performed a screen for mutants of *E. coli* that are deficient in SOS induction in the presence of nalidixic acid. Also, identified a second class of mutants that were either hypersensitive to drug, constitutive for the SOS response, or both.**
- **Performed supplementary tests on the second class of mutants to determine if they were indeed hypersensitive to the drug or constitutive for the SOS response.**
- **Performed an second complete screen directly searching for SOS constitutive mutants.**
- **Performed P1 transductions on mutants that were only picked up once in the screens.**
- **In the process of quantitating the constitutive mutants with liquid *B*-galactosidase assays. Will perform Western blots to confirm overexpression of the recA protein.**

Reportable Outcomes

Presentations

O'Reilly, Erin K., and Kenneth N. Kreuzer. "A novel mutant of T4 topoisomerase that is hypersensitive to multiple classes of antitumor drugs." The Millennial Phage Meeting. McGill University, Montreal, Canada. May 7-11, 2000.

O'Reilly, Erin K., and Kenneth N. Kreuzer. "A novel mutant of T4 topoisomerase that is hypersensitive to multiple classes of antitumor drugs." Phage Meeting 2002. The Evergreen State College, Evergreen, Washington. August, 2001.

O'Reilly, Erin K., and Kenneth N. Kreuzer. "A Unique Type II Topoisomerase Mutant That Is Hypersensitive To A Broad Range of Cleavage-Inducing Antitumor Agents." Era of Hope Department of Defense Breast Cancer Research Program Meeting. Presented at the Young Investigators Luncheon. Orange County Convention Center, Orlando, Florida. September, 2002.

Publications

O'Reilly, Erin K., and Kenneth N. Kreuzer. (2002). A Unique Type II Topoisomerase Mutant That Is Hypersensitive To A Broad Range of Cleavage-Inducing Antitumor Agents. *Biochemistry* **41**:7989-7997.

Conclusions

We have biochemically analyzed two mutants of T4 topoisomerase that display unique properties on their own and interact with one another in an interesting fashion. We first analyzed the G269V single mutant enzyme and found it to be hypersensitive to multiple classes of type II topoisomerase poisons. This mutation is located in a region of the protein that has not been previously implicated in drug or DNA binding. The G269V substitution increases the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. We believe that this is the first type II topoisomerase mutant described that alters drug sensitivity by altering the equilibrium of the enzyme.

It was previously shown *in vivo* that the G269V substitution suppresses the topoisomerase negative phenotype displayed by the S79F mutant strain (ref). We have shown biochemically that the S79F mutant enzyme has a defect in its ATP dependence and this defect is rescued by the G269V substitution. Our preliminary results suggest that this defect is in ATP binding although we have not ruled out a defect in hydrolysis and/or the coupling of these functions to catalysis. Although this defect may enhance drug-resistance the direct cause of resistance is likely an altered drug-binding pocket. Nonetheless, finding mutants that affect ATP dependence in the CAP-like and tower domains is unexpected and has broader implications for enzyme mechanism. Analysis of these mutants has provided a unique perspective on the mechanism of action of topoisomerases and their interactions with inhibitors.

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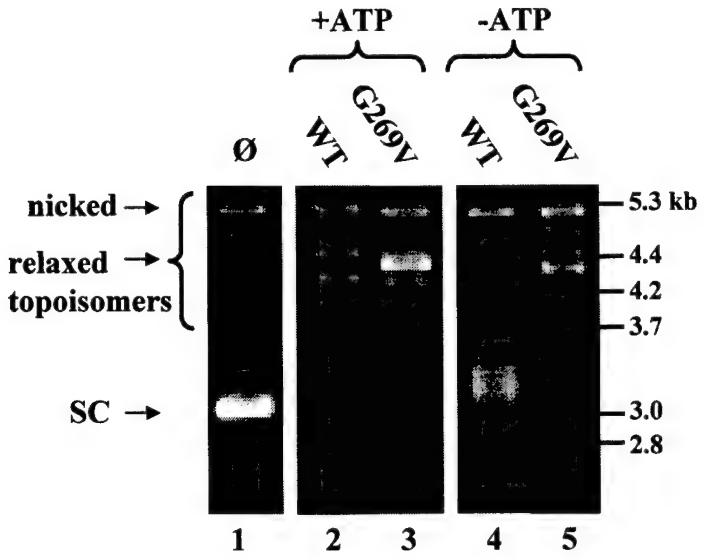

Figure 1. DNA relaxation assays of the wild-type and G269V mutant topoisomerase enzymes in the presence and absence of ATP. Reaction mixtures of 20 μl contained 40 mM Tris-HCL (pH 7.8), 60 mM KCL, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, nuclease-free bovine serum albumin (30 μg/ml), 300 ng of negatively supercoiled pBR322, 25 ng of topoisomerase (enzyme dimer-to-DNA ratio of 1) and either 0.5 mM ATP or no ATP as indicated. The reactions were initiated by the addition topoisomerase, incubated at 30° for 30 min, and then terminated by the addition of 5 μl of gel-loading buffer [5% (wt/vol) SDS, 20% (wt/vol) Ficoll, 0.1% bromophenol blue, and 0.1% xylene cyanol]. Proteinase K (final concentration 100 μg/ml) was then added and the samples were incubated for 1 h at 37° to permit removal of any covalently attached topoisomerase. The reaction products were then resolved by electrophoresis through 0.8% agarose gel. Gels were run in TBE running buffer (89 mM Tris base/89 mM boric acid/ 2.5 mM Na_2EDTA) overnight at 2 V/cm followed by staining with ethidium bromide and visualization with UV illumination. T4 *XbaI*, a size scale (in kb) generated from T4 *XbaI* fragments; Ø, no enzyme, the different forms of DNA are indicated to the left of the figure. The area containing fully relaxed topoisomers is indicated by a bracket. Note that the heavy 4.3 kb band observed in the G269V lanes results from the fact that this enzyme produces ten times more linear DNA than the wild-type enzyme.

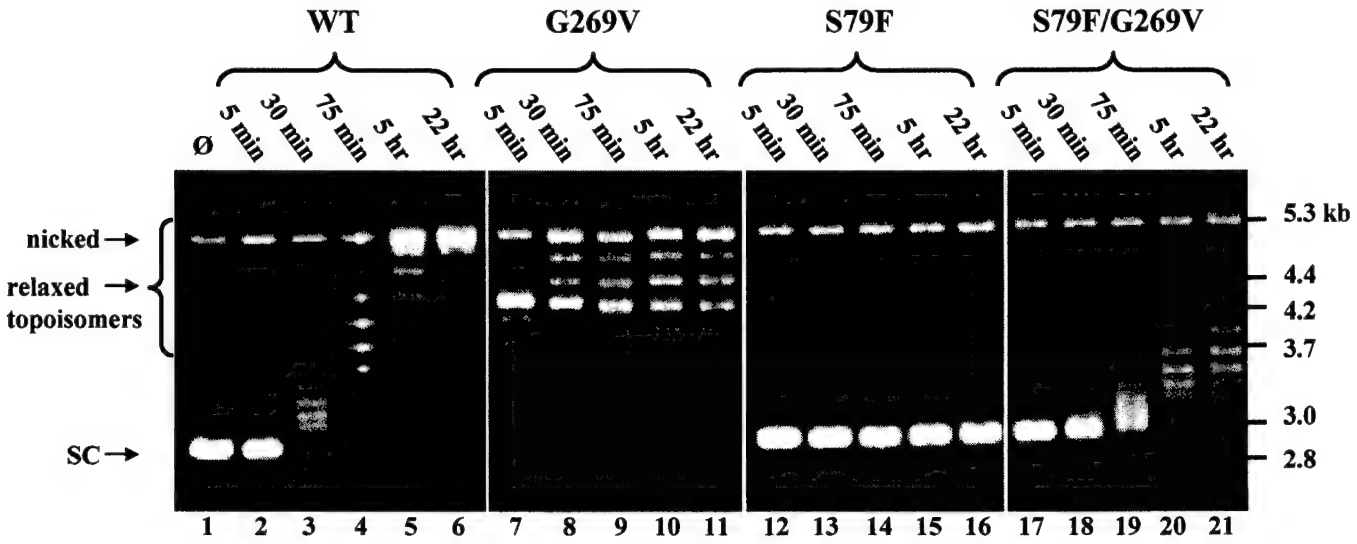

Figure 2. DNA relaxation assays in the absence of ATP over a time course. Four bulk reactions, identical in composition to those described in Figure 1, were created for each of the four enzymes. At the indicated time point a 10 µl sample was removed and terminated immediately by mixing with 2 µl of gel-loading buffer. The samples were then frozen until all of the time points had been taken. The samples were then thawed, digested with Proteinase K, and resolved on 0.8% agarose gels as described in Figure 1. The size scale and abbreviations are the same as described in Figure 1.

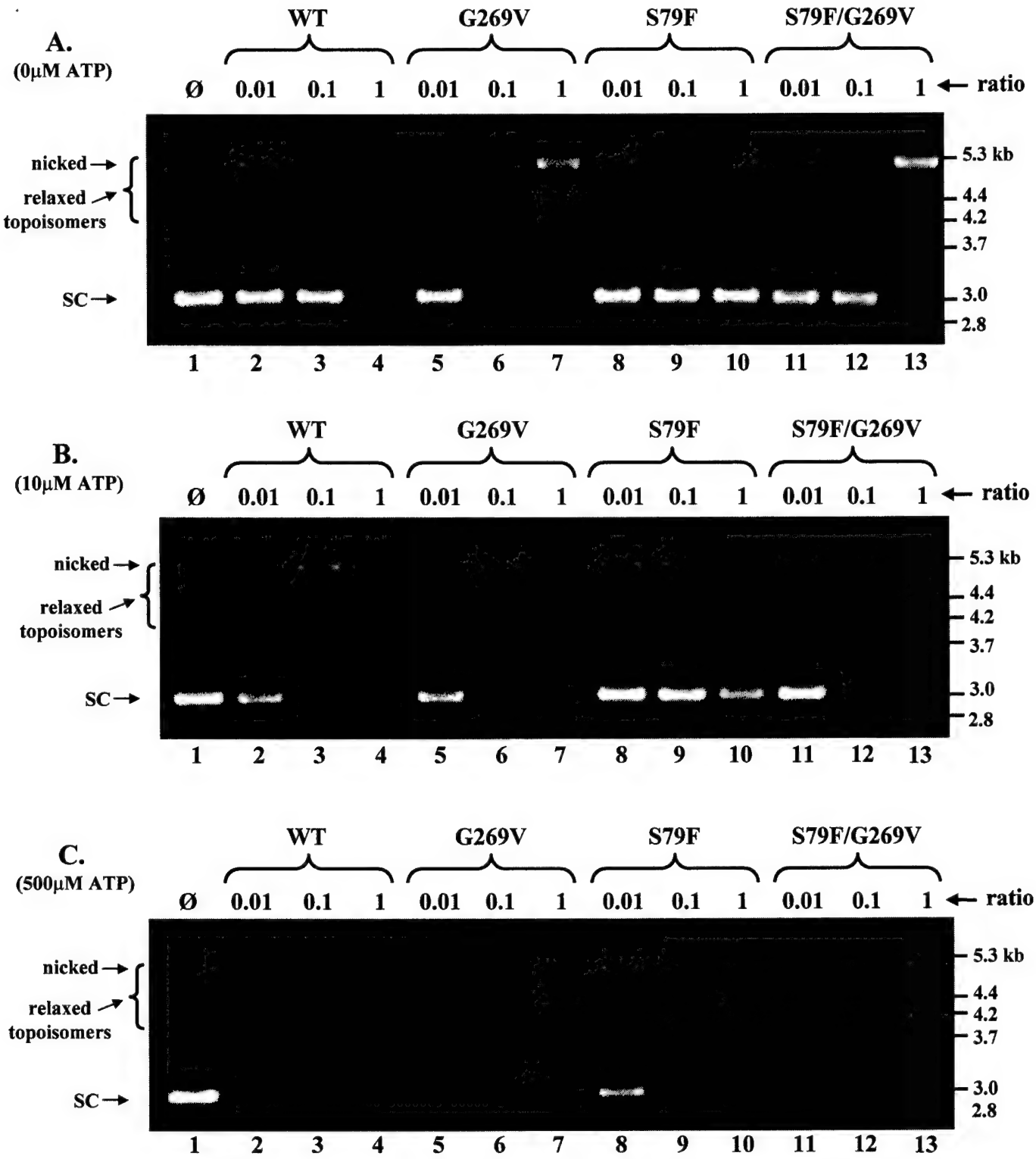

Figure 3. DNA relaxation assays with varying protein and ATP concentrations. Reactions mixtures of 20 μl were identical to those described in Figure 1 accept 0, 10 or 500 μM ATP was used. Also, the amount of enzyme used was varied to yield final topoisomerase dimer-to-DNA ratios of 0.01, 0.1 or 1. The size scale and abbreviations are the same as described in Figure 1.

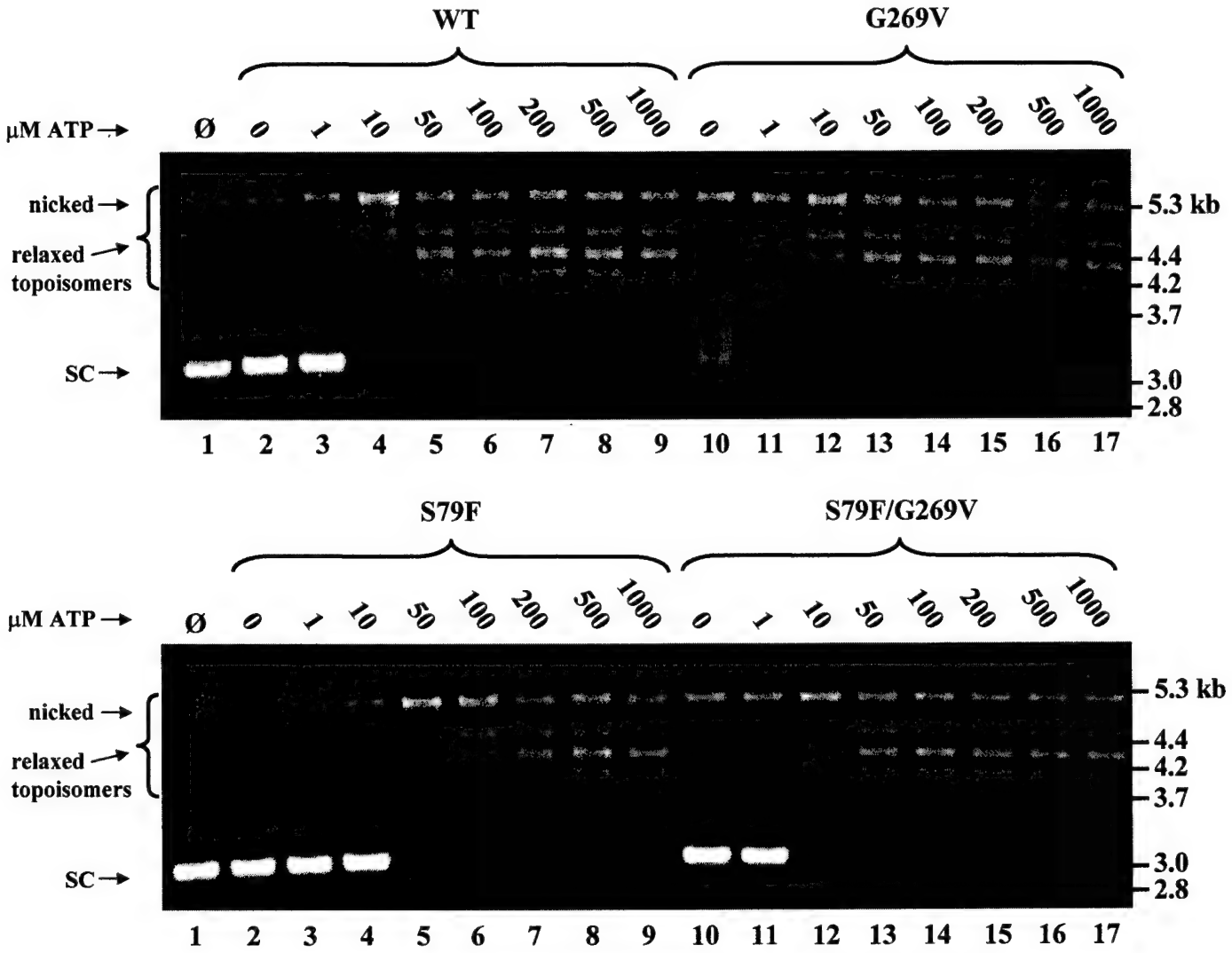

Figure 4. DNA relaxation assays performed over an ATP titration. Reactions mixtures of 20 μl were identical to those described in Figure 1 accept that the enzyme dimer-to-DNA ratio is 0.1 (1 topoisomerase dimer to 10 DNA molecules). ATP concentration are as indicated. The size scale and abbreviations are the same as described in Figure 1.

APPENDIX I

The Isolation of *E. coli* Mutants That Display a Constitutive SOS Phenotype.

Background and Significance

Type II DNA topoisomerases are essential enzymes required for many cellular processes such as DNA replication and transcription (for reviews, see refs *1-4*). These enzyme function by creating a double strand break in one segment of duplex DNA, passing a second duplex segment through this break, and then resealing the break. A critical intermediate in this reaction, the cleavage complex, consists of the protein linked to the 5' ends of the staggered break via phosphotyrosine bonds. A number of important antitumor and antibacterial agents act by stabilizing the cleavage complex intermediate (*3, 5, 6*). Results from numerous systems demonstrate the accumulation of these cleavage complexes to be cytotoxic to cells (*3, 7, 8*).

Although much work has been devoted to understanding how stabilized cleavage complexes cause cytotoxicity, little is known about the specific mechanism. It is clear that recombination repair proteins are involved in processing topoisomerase mediate damage as mutants in this pathway cause hypersensitivity to topoisomerase inhibitors (*9-12*). Thus, in wild-type cells the recombination repair pathway provides some measure of protection against topoisomerase inhibitors. However, it is unclear how the stabilized cleavage complex enters the recombination repair pathway.

Nalidixic acid is a potent inhibitor of bacterial growth and is known to target the bacterial type II topoisomerase, DNA gyrase (*5, 13*). Treatment of cells with nalidixic acid causes the formation of double strand-breaks and induces expression of the SOS regulon. This regulon consists of at least 30 different genes, many of which are involved in the excision and recombination repair pathways (*14, 15*). In the absence of DNA damage, the LexA protein represses the SOS genes and de-repression requires an active RecA protein. The RecA protein becomes activated upon binding single-stranded DNA. In is activated form, RecA causes LexA to proteolytically autocleave at an Ala-Gly bond, which results in de-repression of the SOS regulon (*15*).

The double-strand breaks created following treatment with nalidixic acid are necessary but not sufficient for induction of the SOS response. The multifunctional RecBCD enzyme must process these ends in order to generate the decisive SOS signal, single-stranded DNA (*16*). RecBCD enzyme binds tightly to double-strand DNA ends and unwinds them with its RecB associated helicase activity (*16*). Simultaneously, one of the DNA strands is cleaved with the RecC associated endonucease activity into short single-single stranded fragments. However, only the helicase activity of RecBCD is required for induction of the SOS regulon in response to nalidixic acid (*16*).

It is unclear how the drug stabilized cleavage complex enters the recombinational repair pathway. The original break in the DNA created by the topoisomerase enzyme cannot be the cytotoxic lesion as the 5' ends remain safely linked to protein and are thus

protected from recombination proteins. What then converts these reversible and harmless cleavage complexes into cytotoxic lesions? One possibility is that a specific enzyme recognizes the trapped cleavage complex and converts it into a double strand break. In support of this, a phophodiesterase has been described in some eukaryotic systems that specifically cleaves type I topoisomerases from 3' DNA ends (*17-19*). However, no such enzyme has been described for processing type II topoisomerase 5' linkages.

Another possibility is that the DNA replication machinery is involved in this conversion. It is known that S phase cells are more sensitive to topoisomerase inhibitors than cells in G1 phase and that inhibition of DNA replication with aphidicolin abolishes this sensitivity (*20-23*). Further, drug-stabilized cleavage complexes have been shown to block replication forks *in vivo* and *in vitro* (*24, 25*). Finally, it has been shown that blocked replication forks resulting from mutant helicase proteins lead to the formation of double strand breaks (*26*). These results suggest that the cytotoxicity of the cleavage complex might be dependent upon its ability to stall replication forks.

In order to identify the specific genes required for the repair of topoisomerase cleavage complexes we have performed a screen in *E. coli*. For this screen, we have utilized a strain of *E. coli*, JH39 that has the *lac* operon fused to the promoter of the damage inducible gene, dinD (F', *sfiA*11, *thr*, *leu*, *pro*, *his*, *arg*, *ilv*^{ts}, *gal*, *str*, Pro^{+}, *lacΔ*U169, *dinD*1::Mu dI(Ap^{R} *lac*), see ref *27*). Thus, when the cells are treated with DNA damaging agents the *lacZ* gene expresses the ß-galactosidase enzyme, which can be detected using a number of indicators such as X-gal. Consequently, these cells turn blue in the presence of nalidixic acid on X-gal indicator plates. We constructed a transposon insertion library in JH39 and searched for mutants that were deficient repair by screening for mutants that were hypersensitive to drug and did not turn blue (or were less blue) in the presence of nalidixic acid.

One of the advantages of this screen is that it has some built in positive controls. Namely, we expect to find mutants in *recA* and *recBC* because these proteins are required for recombination repair and have previously been shown to increase drug sensitivity. The RecBCD protein acts at double strand breaks, combining its nuclease and helicase activities to make the DNA single-stranded and accessible to RecA (*16*). However, as stated above it is unclear how a double strand break arises from a trapped cleavage complex. We hope to identify the protein(s) responsible for this conversion. Will we find an endonuclease that specifically recognizes trapped cleavage complexes? Will we instead find an enzyme that specifically recognizes and processes stalled replication forks? A growing number of studies suggest that stalled replication forks may be quite common in growing cells (*16*). Therefore, it seems likely that cells have probably evolved multiple ways of dealing with stalled forks.

Of course, as with any screen, it is possible that we will not find any specific genes that function upstream of RecBCD. There could be redundant pathways for repairing this type of damage and a deficiency in one of them may not be detectable. Further, if the gene(s) of interest are essential we will doubtfully find them in this type of screen. Finally, it is also possible that the mere collision of a replication fork with a

cleavage complex could lead to the formation of a double strand break abrogating the need for any additional proteins. In support of this, it has been shown that a helicase can disrupt the cleavage complex and convert it into an irreversible DNA break *in vitro* (*28*).

In the context of cancer chemotherapy, it is important to distinguish between the above possibilities. Identification of the specific proteins and steps involved in this repair pathway is the first step in finding specific inhibitors of repair. These types of inhibitors could be used to in combination with the currently available topoisomerase inhibitors to enhance their activity.

Summary of Results

An undergraduate member of the laboratory, Katie Newmark, began this screen and identified a collection of mutants that were either light blue or white in the presence of nalidixic acid. She screened approximately 18,000 colonies and is currently working with a collection of roughly 30 mutants. Although her specific results will not be discussed here, she did find mutants in the *recA* and *recBC* genes, as expected.

It became clear from the first day of screening that there was a class of mutants that we had not predicted, namely those that were darker blue than the wild-type strain. There were two obvious, non-mutually exclusive, causes of this dark blue phenotype. Certainly some of these dark blue colonies were mutants in drug permeability or efflux mechanisms. Thus, there would be an effectively higher concentration of drug inside the cells leading to the darker blue color. A more intriguing possibility was that some of the mutants were constitutively expressing the SOS regulon. In this initial screen, we identified a total of 72 dark blue mutants.

It seemed prudent to pursue this dark blue class of mutants for two reasons. For one thing, a systematic screen for constitutive SOS mutants has never been performed. This type of screen would almost certainly lead to a very interesting collection of hyper-recombinagenic mutants. These types of mutants could be relatives of cancer genes in higher eukaryotic organisms (see below). Secondly, the gene(s) involved in the repair of topoisomerase mediate damage could be SOS constitutive and therefore might be hidden in this class of mutants.

In order to differentiate between drug efflux/pump mutants and SOS constitutive mutants we plated each of the 72 mutants onto plates containing X-gal and no drug and compared them to the wild-type strain. We found 21 of the mutants did not turn blue in the absence of drug. These are almost certainly mutants in drug permeability or efflux mechanisms. These mutants should have a generic hypersensitivity phenotype and we would expect them to be hypersensitive to drugs that are not topoisomerase inhibitors. We will test these mutants with a variety of drugs such as tetracycline before finally eliminating them.

Of the 51 remaining SOS constitutive mutants, 14 of them were also found to be hypersensitive to nalidixic acid by using drug disc tests (data not shown). These 51

mutants were also qualitatively classified as being either strongly or partially constitutive based on the intensity of blue color observed on plates containing X-gal. Thus, we had four different classes of mutants, those that were strongly constitutive and either hypersensitive (4) or not (22) and partially constitutive and either hypersensitive (10) or not (15).

Although we felt this was a fairly solid set of constitutive mutants they were obtained circuitously from a screen with a different purpose. We wondered if we might have missed some genes by using this indirect method of screening? Thus, we decided to perform a second screen whereby we directly searched for constitutive SOS mutants. We again created a transposon insertion library and screened an additional 15,000 colonies for mutants that turned blue on plates containing X-gal. From this screen we obtained an additional 111 mutants. We performed drug sensitivity tests on the total set of mutants from both screens and scored them as being either strongly or partially constitutive and either hypersensitive or not to drug. We purified genomic DNA from each of these 162 mutants and sequenced them directly using a primer located at the 3' end of the transposon. From the sequencing and mapping we had hits in 64 genes, however, 37 of these genes had only been hit one time. We were concerned that the constitutive phenotype in some of these 37 mutants might be caused by a mutation at a second site. We therefore performed P1 transductions on each of these 37 mutants and found that the constitutive phenotype did not transduce in 12 of them leaving us with 52 constitutive SOS genes. These genes are summarized in Tables 1 and 2.

We are in the process of quantitating these mutants with liquid β-galactosidase assays. We will also validate their SOS constitutive nature by performing Western blots to confirm over-expression of the RecA protein. Of special interest are those mutants that are hypersensitive to drug such as *ruvABC* and *uvrD*. We wonder if these mutants further induce the SOS response to nalidixic acid treatment? If not, they could be mutants that are involved in the repair of topoisomerase-mediated damage and/or the repair of stalled forks.

Relevance to Cancer Research

The results from this screen will have many applications to cancer research and therapy. It seems clear that the repair of topoisomerase-mediated damage somehow involves the recombination repair pathway and thus, double strand breaks. This overt break in the DNA must be repaired properly or genome rearrangements could occur. In fact, the generation of secondary tumors after treatment with antitumor agents could be related to these types of repair failures. Obviously, any specific genes we identify as being important for the repair of topoisomerase-mediated damage could be used as new drug targets.

The identification of mutants with a SOS constitutive phenotype will also be applicable to cancer research. Normally, the SOS response is tightly regulated event because uncontrolled expression is highly mutagenic and can lead to genome rearrangements. Mutants that express this regulon in a constitutive manner will not be

protected from these high rates of mutation. Some of genes we have isolated have relatives in eukaryotic organisms that are thought to be cancer genes. For example, we have identified a number of helicases as constitutive SOS mutants and a number of human cancers are associated with mutant helicase proteins (e.g. Werner, Bloom and Rothmond-Thomson syndromes, *29-32*). These and possibly other genes from this screen could be used as simple models for higher eukaryotic systems.

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Table 1. Genes of *E. coli* that when dirupted give a stongly constitutive SOS phenotype.

Strong	HS?[1]	Function
lexA	No	SOS regulon repressor
rep	No	helicase
rpoZ	No	RNA-polymerase subunit (omega)
xerC	No	site specific recombinase
xerD	No	site specific recombinase
ftsK	No	SpoIIIa like function/cell division
dam	No	DNA adenine methylase
damX	No	inhibits cell division
yehB	No	*homology*-fimbrial usher protein
spoT	No	magic spot synthetase
purE	No	catalytic subunit of phosphoribosylaminoimidazole carboxylase
tynA	No	tyramine oxidase (or copper amine oxidase)
folK	No	7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase
dnaQ	No	3'-5' exonuclease subunit of DNA polymerase III
b1721	No	*homology* -ankrin repeat protein
dcd	No	2'- deoxycytosine deaminase
purF	No	glutamine phosphoribosylpyrophosphate amidotransferase
yggM	No	*homology* - alpha helix chain
trpB	No	tryptophan synthase (B-chain)
yebC	No	*homology* - unknown
cvpA	No	colicin-related functions (colicin V)
thyA	Yes	thymidylate synthetase
ruvA	Yes	helicase subunit involved in branch migration of holiday junctions (subunit B)
ruvB	Yes	helicase subunit involved in branch migration of holiday junctions (subunit A)
ruvC	Yes	endonuclease resolves holiday structures
tdcE	Yes	formate acetyltransferase
polA	Yes	DNA polymerase I

[1]Drug sensitivity was determined by using Etest strips from AB Biodisk. These strips contained an exponential gradient of nalidixic acid. Drug hypersensitivity was assigned if the growth inhibitory concentrations of the mutants were less than those of the wild-type strain, JH39.

Table 2. Genes of *E. coli* that when dirupted give a partial constitutive SOS phenotype.

Partial	HS?[1]	Function
ompA	No	outer membrane protein 3a
htrA (degP)	No	periplasmic degradative protease
sppA	No	signal peptide protease (protease IV)
surA	No	survival protein-chaperone/folding catalyst
recG	No	helicase - resolves holiday junctions
gmhA	No	phophoheptose isomerase
yibP	No	*homology*-membrane protein
yhcB	No	*homology*-putative periplasmic protein
yigF/yigG	No	*homology*-putative inner membrane proteins
b2513 (yfgM)	No	*homology*- hypothetical protein with putative transmembrane region
sfhB/b2595[2]	No	sfhB pseudouridine synthase D; *b2595 homology* - lipoprotein/ competence protein
b1450 (yncC)	No	*homology* - transcription regulator
yejM	No	*homology* - sulfatase
folA	No	dihydrofolate reductase
recN	No	replication repair gene
dsbB	No	reoxidizes DsbA (DsbA forms disulfide bond in P-ring of flagella)
purA	R	adenyl succinate syhtetase
purL	R	phophoribosylformylglycinamidine (FGAM) synthase
acrA	Yes	component of acrAB efflux pump
acrB	Yes	component of acrAB efflux pump
tolC	Yes	outer membrane efflux pump
uvrD	Yes	DNA dependent helicase II
ftsX	Yes	inner membrane protein; interacts with ftsE may form ABC-transporter
ftsE	Yes	dimer associates with ftsX to form possible ABC-transporter.

[1]Drug sensitivity was determined by using Etest strips from AB Biodisk. These strips contained an exponential gradient of nalidixic acid. Drug hypersensitivity or resistance was assigned if the growth inhibitory concentrations of the mutants were less than or greater than those of the wild-type strain, JH39.

[2]The transposon insertion lies in between these two genes.

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A Unique Type II Topoisomerase Mutant That Is Hypersensitive to a Broad Range of Cleavage-Inducing Antitumor Agents†

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ABSTRACT: Bacteriophage T4 provides a useful model system for dissecting the mechanism of action of antitumor agents that target type II DNA topoisomerases. Many of these inhibitors act by trapping the cleavage complex, a covalent complex of enzyme and broken DNA. Previous analysis showed that a drug-resistant T4 mutant harbored two amino acid substitutions (S79F, G269V) in topoisomerase subunit gp52. Surprisingly, the single amino acid substitution, G269V, was shown to confer hypersensitivity in vivo to *m*-AMSA and oxolinic acid [Freudenreich, C. H., et al. (1998) *Cancer Res. 58*, 1260−1267]. We purified this G269V mutant enzyme and found it to be hypersensitive to a number of cleavage-inducing inhibitors including *m*-AMSA, VP-16, mitoxantrone, ellipticine, and oxolinic acid. While the mutant enzyme did not exhibit altered DNA cleavage site specificity compared to the wild-type enzyme, it did display an apparent 10-fold increase in drug-independent DNA cleavage. This suggests a novel mechanism of altered drug sensitivity in which the enzyme equilibrium has been shifted to favor the cleavage complex, resulting in an increase in the concentration of cleavage intermediates available to inhibitors. Mutations that alter drug sensitivities tend to cluster within two specific regions of all type II topoisomerases. Residue G269 of gp52 lies outside of these regions, and it is therefore not surprising that G269V leads to a unique mechanism of drug hypersensitivity. We believe that this mutant defines a new category of type II topoisomerase mutants, namely, those that are hypersensitive to all inhibitors that stabilize the cleavage complex.

Type II DNA topoisomerases are essential enzymes required for many cellular processes such as DNA replication and transcription (for reviews, see refs *1*−*4*). These enzymes function by creating a double strand break in one segment of duplex DNA, passing a second duplex segment through this break, and then resealing the break. A critical intermediate in this reaction, the cleavage complex, consists of the protein linked to the 5′ ends of the staggered break via phosphotyrosine bonds.

In addition to their essential roles in cellular metabolism, type II topoisomerases are the targets of many clinically important antibacterial and antitumor agents. The prokaryotic type II topoisomerases (DNA gyrase, topoisomerase IV) are the targets of the important broad spectrum antibacterial quinolones and fluoroquinolones (*5*), while the eukaryotic type II topoisomerases are inhibited by several classes of antitumor agents, including the acridines, anthracyclines, ellipticines, and epipodophyllotoxins (for reviews, see refs *3* and *6*). These drugs act by stabilizing the cleavage complex rather than by simply inhibiting strand passage (for reviews, see refs *3*, *7*, and *8*). Direct binding studies from a number of systems support the existence of a stable enzyme−DNA−drug ternary complex (*9*−*11*; S. Neece and K. Kreuzer, unpublished data). The stabilized cleavage complex itself is relatively harmless to cells, but it can be converted into potentially lethal DNA damage by cellular processes such as DNA replication (*12*−*14*).

In the context of antitumor drug action, bacteriophage T4 provides a useful and valid model system. The T4 type II topoisomerase is a multisubunit enzyme composed of the products of T4 genes *39*, *52*, and *60* (*15*, *16*). Gp39[1] and gp60 are homologous to portions of *Escherichia coli* GyrB and to the amino-terminal half of eukaryotic topoisomerase II while gp52 contains the active site tyrosine and is homologous to *E. coli* GyrA and the carboxy-terminal half of the eukaryotic enzymes. The phage-encoded enzyme is sensitive to many of the same antitumor agents that inhibit the eukaryotic enzyme and is partially sensitive to at least one of the antibacterial quinolones (*17*, *18*). The straightforward genetics, biochemistry, and molecular biology of the phage T4 system have facilitated studies on the mechanism of action of these topoisomerase inhibitors.

The inhibitor binding site within the cleavage complex consists of both protein and DNA. Involvement of the topoisomerase in drug binding is indicated by the fact that

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[1] Abbreviations: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide, *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; 2-Me-9-OH-E$^+$, 2-methyl-9-hydroxyellipticinium acetate; VP-16, etoposide or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-*β*-D-glucopyranoside); VM-26, teniposide or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-*β*-D-glucopyranoside); gp, gene product; NC, nitrocellulose; NY, nylon.

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single amino acid changes in specific regions of the enzyme can confer altered drug sensitivities (reviewed in ref *19*). For example, the yeast topoisomerase S741W mutant is hypersensitive to VP-16 but resistant to the fluoroquinolone CP-115,953 (*20*). In T4, the E457K substitution in gp39 causes resistance to a number of drugs but hypersensitivity to oxolinic acid and VP-16 (*21*, *22*). The differential effects of these mutations on different drug families imply that specific amino acid residues of the protein interact with the drugs.

Involvement of DNA in the drug binding site was originally proposed on the basis of early experiments that revealed distinct patterns of cleavage sites in the presence of different drug classes (*17*, *23*−*26*). Consensus sequences derived with eukaryotic enzymes suggested that the base pairs immediately flanking the cleavage site determined which drug could trap the enzyme at a particular cleavage site (*26*−*28*). The detailed mutational analysis of one particular T4 topoisomerase cleavage site provided direct evidence that the base pair immediately 5′ of the break confers the drug site preference (*29*). The preferred base pairs for the T4 enzyme paralleled those of the eukaryotic enzymes, suggesting that the same rules govern inhibitor binding specificity in evolutionarily distant topoisomerases (*29*). Further, using the T4 system, the inhibitor was precisely localized to the sites of DNA cleavage using a photoactivatable analogue of *m*-AMSA (*30*). Thus, the inhibitors bind directly at the sites of DNA cleavage (perhaps by intercalation) and act by preventing religation of the two strands and/or enhancing the forward rate of cleavage.

Analysis of a T4 gene *52* mutant led to some interesting and unexpected results. The mutant strain was originally isolated from a selection for *m*-AMSA-resistant phage mutants (*31*). The mutant was found to harbor two amino acid substitutions: S79F and G269V (*22*). The S79F mutation was quite provocative because the corresponding residue from bacterial DNA gyrase (A84) was known to be a hot spot for drug resistance mutations (*9*). Surprisingly, when the S79F mutation was substituted into a wild-type background, the resulting phage behaved as topoisomerase negative in vivo (*22*). It was hypothesized that the G269V mutation suppresses the defect caused by the substitution at residue S79. Indeed, when the double mutant (S79F/G269V) was substituted into a wild-type background, the resulting phage behaved as topoisomerase proficient and displayed the same drug-resistant phenotype as the original mutant.

Remarkably, the G269V single mutant phage exhibited hypersensitivity to *m*-AMSA and oxolinic acid in vivo (*22*). This hypersensitive phenotype cannot be readily explained by the location of this residue in the protein as none of the corresponding residues from the yeast or bacterial enzymes have been implicated in drug binding. Sequence comparisons with the yeast enzyme place residue G269 in the "tower domain" of the protein, which lies outside the two domains where drug resistance mutations cluster (see Discussion). Furthermore, there is no biochemical or structural evidence for involvement of the tower domain in DNA binding. Therefore, it is unclear how the G269V substitution causes drug hypersensitivity or suppresses the defect at position S79.

In the present study, we have biochemically analyzed the G269V enzyme and found it to be hypersensitive to a broad range of type II topoisomerase inhibitors. While the G269V mutant enzyme did not exhibit altered cleavage site specificity compared to the wild-type enzyme, it did display elevated levels of drug-independent DNA cleavage. This suggests a novel mechanism for altered drug sensitivity, namely, an alteration in the reaction pathway that leads to an increase in the frequency of cleavage complexes available to inhibitors.

EXPERIMENTAL PROCEDURES

Materials. *m*-AMSA (NSC 249992), ellipticine (NSC 71795), and mitoxantrone (NSC 299195) were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. 2-Me-9-OH-E^+ (NSC 264137) was a generous gift of Dr. C. Paoletti (Institute Gustave-Roussy, Villejuif). VM-26 (NSC 122819) and VP-16 (NSC 141540) were kindly provided by Bristol-Myers Pharmaceutical Co. (Wallingford, CT). Oxolinic acid and additional VP-16 were purchased from Sigma. 2-Me-9-OH-E^+ was dissolved in water, oxolinic acid in 50 mM NaOH, and all other drugs in DMSO. Immediately prior to use, an aliquot was diluted with water to the required concentration. Final reaction concentrations of DMSO and NaOH were <1.5% and <0.5 μM, respectively, and did not significantly affect DNA cleavage levels (data not shown).

Plasmid pBR322 was purified from *E. coli* DH5α using an alkaline lysis procedure (*32*) followed by cesium chloride/ethidium bromide isopycnic centrifugation. Restriction enzymes were obtained from New England Biolabs.

T4 Topoisomerases. Wild-type and G269V mutant T4 topoisomerases were purified by adapting the T7 expression system for use in phage T4, as originally described by Singer and Gold (*33*). As will be described in more detail elsewhere, a T7 promoter was placed upstream of each T4 topoisomerase gene (*39*, *52*, *60*) to create the overproduction phage strain for wild-type enzyme (K. Magee and K. Kreuzer, unpublished data). We then crossed the G269V mutation into this background to create the overproduction strain for the mutant enzyme. *E. coli* cells were induced for T7 RNA polymerase prior to infection with each of the topoisomerase overproduction strains, and T4 topoisomerase was purified as previously described (*34*, *35*). Protein concentrations were determined using the Bradford assay (*36*) as described previously (*31*). Enzymatic activities were determined using a standard DNA relaxation assay (*35*).

End Labeling of DNA Substrates. pBR322 DNA was linearized with *Eco*RI, and then the recessed 3′ termini were filled in using the Klenow exo$^-$ DNA polymerase I fragment in the presence of either [α-^{33}P]- or [α-^{32}P]dATP and dTTP, yielding a 4361 bp substrate labeled at each *Eco*RI terminus. When indicated, the labeled substrate was further digested with *Hin*dIII to yield a 4336 bp fragment labeled at only one end.

DNA Cleavage Assays. Reaction mixtures (20 μL) contained 40 mM Tris-HCl (pH 7.8), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na_2-EDTA, nuclease-free bovine serum albumin (30 μg/mL), and the specified amounts of DNA substrate, topoisomerase, and inhibitor. Cleavage reactions were performed at an approximate ratio of one topoisomerase dimer to one molecule of DNA except where indicated (see figure legends). The reactions were initiated by the addition of

topoisomerase, incubated at 30 °C for 30 min, and terminated by the addition of 5 μL of gel-loading buffer [5% (w/v) SDS, 20% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol]. Proteinase K (final concentration 100 μg/mL) was added, and the samples were incubated for 1 h at 37 °C to remove covalently attached topoisomerase. The reaction products were resolved by electrophoresis through either 0.8% agarose gels or 6% polyacrylamide gels in TBE running buffer (89 mM Tris base/89 mM boric acid/2.5 mM Na_2EDTA). Agarose gels contained ethidium bromide (2.5 μg/mL) and were run overnight at 2 V/cm; DNA was visualized by ethidium bromide fluorescence and quantitated using an Alpha Innotech digital imaging system. Polyacrylamide gels were run at 30 W for approximately 6 h. After gel drying, the labeled DNA was visualized by autoradiography on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Filter Binding Method. The levels of DNA cleavage were quantitated using a nitrocellulose filter-binding assay similar to that described by Wong and Lohman (*37*). A Millipore vacuum pressure pump (Fisher, XX5500000) was used at 500−600 mm Hg during filtration on a 96-well dot-blot apparatus (Minifold I, Schleicher and Schuell). Nitrocellulose (NC; Schleicher and Schuell, BA-85) was used as the top filter to trap the protein-linked DNA, and nylon (NY; NEN, NEF-994) was used as the second filter to trap the protein-free DNA that does not bind to the NC filter. A single sheet of gel blot paper (Schleicher & Schuell) was used as a third layer to reduce lateral diffusion of unbound DNA. The NC and NY filters were equilibrated in 1× filter buffer [50 mM Tris-HCl (pH 7.8), 200 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_2EDTA] for 30 min before use, and the gel blot paper was immersed in the same buffer prior to use.

Approximately 1−2 ng (10000 cpm) of linearized DNA labeled at both ends with [α-^{33}P]dATP and 100 ng of linearized unlabeled pBR322 DNA were combined with 9.2 ng (35 fmol) of topoisomerase (molar enzyme dimer-to-DNA ratio of 1; except where indicated). The reactions were performed as described above (see DNA Cleavage Assays) except that they were terminated by the addition of 5 μL of 1% (w/v) SDS and were not treated with proteinase K. After termination, 15 μL of 5× filter buffer and 50 μL of water were added to each reaction. Immediately prior to loading, the wells were flushed with 400 μL of 1× filter buffer under vacuum. The samples were loaded without vacuum, the vacuum was reapplied, and the wells were then washed twice with 400 μL of 1× filter buffer. The two filters were then blotted dry, wrapped in plastic wrap (Mylar), and imaged by autoradiography on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The "dots" were quantitated using the ImageQuant software provided by the manufacturer. The percent cleavage was calculated from the ratio of counts on the NC filter to the total counts (NC filter + NY filter). This percent cleavage was corrected by subtracting the small amounts (1−2%) of protein-free DNA that bind to the NC filter in control reactions. The amount of drug-dependent cleavage was determined by subtracting the amount of drug-independent cleavage observed for each enzyme, leading to no apparent cleavage at 0 μM drug. It is important to note that the G269V enzyme yields approximately 10% cleavage in the absence of drug at a molar enzyme dimer-to-DNA ratio of 1, while the wild-type enzyme generates about 1% cleavage (see below).

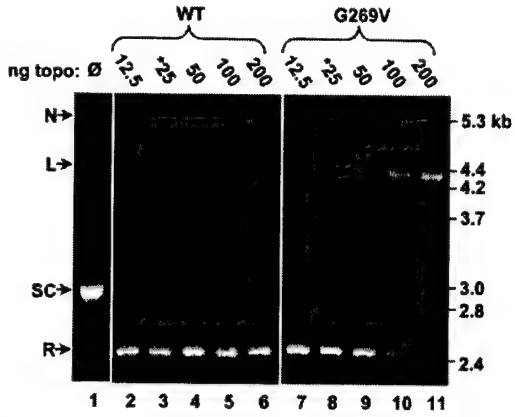

FIGURE 1: DNA cleavage assay with negatively supercoiled pBR322 DNA. Drug-independent DNA cleavage levels of the wild-type and G269V mutant enzyme were compared in a protein titration. Reactions contained 300 ng (107 fmol) of DNA and the indicated amounts of protein. The asterisks indicate 96 fmol of enzyme, for a molar enzyme dimer-to-DNA ratio of 0.9. A size scale (kb, kilobases), generated from the migration of T4dC *Xba*I restriction fragments, appears in the right-hand margin, and different forms of DNA are indicated in the left-hand margin. Abbreviations: N, nicked DNA; L, linear DNA; SC, supercoiled DNA; R, relaxed DNA; ϕ, a control reaction with no enzyme.

A final correction was also applied to account for protein that is linked to unlabeled DNA as a result of multiple cleavage events. The substrate is labeled at each end, so one cleavage event per DNA molecule results in both topoisomerase monomers being linked to a labeled DNA fragment. However, any additional cleavage events on the same molecule of DNA would not generate additional signal. Therefore, assuming that cleavage events are randomly distributed, the data were corrected with the following equation derived from the Poisson distribution

$$C_t = -\ln(1 - C_o)$$

where C_o is the experimentally observed amount of labeled DNA cleavage and C_t is the predicted amount of total DNA cleavage. For example, 30% observed cleavage ($C_o = 0.3$) is reported as 35.7% total cleavage ($C_t = 0.357$). The averages of triplicate measurements are shown with error bars indicating standard deviations.

RESULTS

The G269V Substitution Causes Increased Levels of Drug-Independent DNA Cleavage. The G269V substitution caused dramatic hypersensitivity in vivo to both *m*-AMSA and oxolinic acid (*22*). In our system, these are the only two inhibitors that can be analyzed in vivo. We therefore purified the mutant enzyme so that we could test its sensitivity to other cleavage-inducing inhibitors. The G269V mutant topoisomerase displayed a specific activity similar to that of the wild-type protein (2.0×10^6 and 2.9×10^6 units/mg, respectively; data not shown). However, in the absence of drug, the G269V mutant protein caused the formation of much larger amounts of linear DNA from a circular substrate than did the wild-type enzyme (Figure 1, compare lanes 2−6 with lanes 7−11). At an approximate molar enzyme dimer-

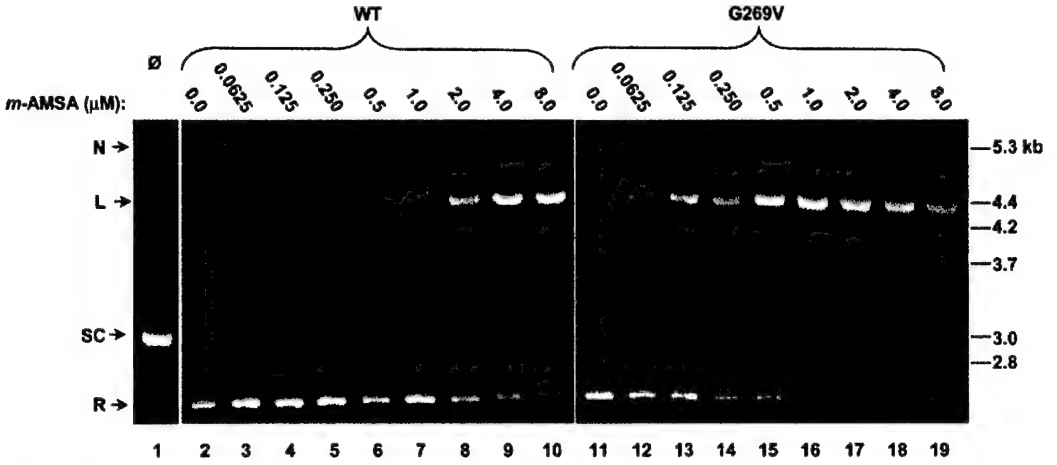

FIGURE 2: DNA cleavage assay with negatively supercoiled pBR322 DNA in the presence of *m*-AMSA. The *m*-AMSA sensitivities of the wild-type and G269V enzymes were compared in a drug titration. Reactions contained 25 ng of protein (96 fmol), 300 ng of DNA (107 fmol), and the indicated concentrations of *m*-AMSA (micromolar). The size scale and abbreviations are the same as described in Figure 1 except that ϕ denotes a control reaction with no enzyme and no drug.

to-DNA ratio of 1, we estimate that 10% of the DNA was in a cleavage complex with the G269V mutant enzyme while the amount of DNA in a cleavage complex with the wild-type enzyme was too low for accurate quantitation (Figure 1, lanes 3 and 8). These cleavage products were protein linked, since the linear DNA became a broadened band of decreased electrophoretic mobility in the absence of proteinase K digestion (data not shown). Protein-linked DNA products are a normal short-lived intermediate in the topoisomerase relaxation reaction, but our results show that the G269V mutant topoisomerase accumulates higher than normal amounts of this intermediate.

The G269V Substitution Causes in Vitro Hypersensitivity to m-AMSA. We next tested whether the purified G269V enzyme displayed *m*-AMSA hypersensitivity, as was observed in vivo. As expected from the in vivo results, the G269V protein produced more linear DNA at relatively low levels of *m*-AMSA than did the wild-type enzyme (Figure 2). This conclusion is somewhat obscured by the high levels of drug-independent cleavage observed with the G269V enzyme (Figure 2, compare lane 2 to lane 11). Nonetheless, the drug-dependent percent cleavage can be determined by subtracting the drug-independent percent cleavage. Thus, at 0.125 μM *m*-AMSA, the G269V mutant enzyme yielded about 6-fold more drug-dependent cleavage than the wild-type enzyme (Figure 2, lanes 4 and 13). This difference becomes less apparent at higher drug levels presumably because the drug concentrations are reaching saturation. Additionally, at high drug levels, multiple cleavage events are evident from the smearing and minor cleavage products (Figure 2, lanes 8−10 and 15−19). All of these *m*-AMSA-induced cleavage products were shown to be protein linked by omitting the proteinase K digestion (data not shown). Our results show that the purified G269V mutant topoisomerase is indeed hypersensitive to *m*-AMSA with respect to cleavage complex formation, mirroring the in vivo hypersensitive phenotype of the G269V mutant phage.

The G269V Substitution Causes in Vitro Hypersensitivity to Multiple Classes of Type II Topoisomerase Inhibitors. We next analyzed DNA cleavage of the G269V mutant using a quantitative approach. We employed a filter-binding method similar to one described by Wong and Lohman (*37*), using a 96-well dot-blot apparatus with a double filter. After the reactions were stopped with SDS, protein-linked DNA molecules were trapped on a NC filter while free DNA passed through and bound to an underlying NY filter. This enables a direct determination of percent cleavage of the labeled substrate, with triplicate repeats of 20 different reactions analyzed on a single filter.

This filter-binding method allowed a more accurate comparison of drug-independent DNA cleavage levels between the wild-type and G269V mutant enzymes. We determined enzyme dimer-to-DNA ratios that produced similar levels of total cleavage. With the G269V enzyme, 10.6% (±3.0; $n = 7$) of the DNA was in a cleavage complex at an enzyme dimer-to-DNA ratio of 1, while an enzyme dimer-to-DNA ratio of 10 was needed to trap 8.0% (±0.7%, $n = 3$) of the DNA in a cleavage complex with the wild-type enzyme. Thus, the G269V mutant enzyme is detected in cleavage complexes at roughly 10 times the level of the wild-type enzyme.

Again using the filter-binding method, we compared the DNA cleavage levels of the wild-type and G269V mutant topiosomerases induced by five different drug classes. Drug-dependent cleavage levels were determined by subtracting the drug-independent cleavage levels from each drug titration (enzyme dimer-to-DNA ratio of 1). Seven topoisomerase inhibitors were tested: ellipticine and 2-Me-9-OH-E$^+$ (ellipticines), mitoxantrone diacetate (substituted anthraquinone), VM-26 and VP-16 (epipodophyllotoxins), oxolinic acid (quinolone), and the previously assayed *m*-AMSA (an aminoacridine).

The results obtained with *m*-AMSA (Figure 3) can be compared directly with the DNA cleavage assay above (Figure 2). The filter-binding assay showed that the G269V enzyme enhanced drug-dependent cleavage, especially at low *m*-AMSA concentrations. At 0.125 μM *m*-AMSA, there is almost 10 times more drug-dependent cleavage with the G269V enzyme than with the wild-type enzyme (Figure 3, 7.6% versus 0.77%). As observed with the gel assays, this margin is narrowed as the drug reaches saturating levels. Thus, the filter-binding method can accurately measure drug

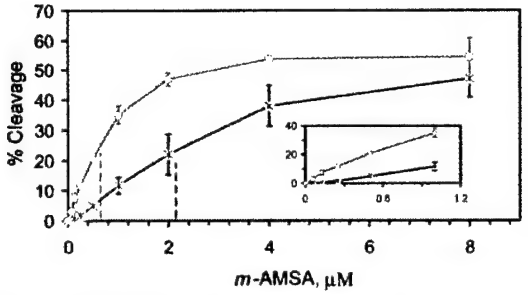

FIGURE 3: Quantitation of DNA cleavage in the presence of *m*-AMSA. A nitrocellulose filter-binding assay similar to that of Wong and Lohman (*37*) was used to assess the sensitivities of the wild-type and mutant enzymes to *m*-AMSA. Cleavage reactions contained a small amount (~1−2 ng) of ^{33}P-end-labeled pBR322 DNA, 100 ng of unlabeled linear pBR322 DNA, 9.2 ng of either wild-type or mutant enzymes (35 fmol; molar enzyme dimer-to-DNA ratio of 1), and the indicated concentrations of *m*-AMSA. The data represent an average of three independent experiments. Percent cleavage has been corrected for drug-independent cleavage levels and for multiple cleavage events using the Poisson distribution (see Experimental Procedures). Key: (○) G269V; (×) wild type.

Table 1: Drug Sensitivities of the Wild-Type and G269V Mutant Enzymes

drug	[drug] for half-maximal cleavage (μM)[a]		HS factor[b]
	WT	G269V	
m-AMSA	2.2	0.75	2.9
ellipticine	1.3	0.4	3.3
2-Me-9-OH-E$^+$	0.3	0.19	1.6
VP-16	29.2	11.1	2.6
VM-26	27	13.3	2.0
mitoxantrone	0.052	0.034	1.5
oxolinic acid	255	85	3.0

[a] Derived from Figures 3 and 4. [b] Calculated by dividing the wild-type (WT) half-maximal [drug] by the G269V half-maximal [drug].

sensitivity over a broad range of drug concentrations and verifies the *m*-AMSA hypersensitivity of the G269V enzyme.

Somewhat surprisingly, the G269V mutant topoisomerase was found to be hypersensitive to all of the drugs tested (Figure 4). All the curves follow the same general pattern, with more dramatic sensitivity at lower drug concentrations (see inserts). To compare the relative levels of sensitivity, we determined the drug concentrations required for half-maximal cleavage and calculated an overall hypersensitivity factor of G269V enzyme compared to wild type (Table 1). According to this "HS factor", the mutant enzyme was between 1.5- and 3.3-fold more sensitive to all of the drugs, with no obvious difference between intercalators (ellipticines, mitoxantrone, and *m*-AMSA) and nonintercalators (oxolinic acid and epipodophyllotoxins). These results suggest that the G269V mutant topoisomerase is responding to all of the drugs in a similar manner.

The G269V Substitution Does Not Alter the Site Specificity of the Enzyme in the Absence or Presence of Drugs. The T4 type II topoisomerase cleaves duplex DNA at specific sites, generating a reproducible pattern of cleavage fragments from a given DNA substrate. Previous work has shown that each chemical class of inhibitor exerts a unique effect on the DNA cleavage site specificity of the wild-type T4 or eukaryotic enzyme (*17*, *23*−*26*, *29*). Further, many previously isolated topoisomerase mutants that alter drug sensitivity also alter the DNA site specificity of the enzyme, presumably because the drug binding pocket of the enzyme is very close to the DNA (*17*, *23*−*26*, *38*; also, see the introduction). To see if the G269V mutation alters the DNA site specificity and, by implication, the drug binding pocket, we performed DNA cleavage assays in the presence and absence of drugs using a linear substrate that was labeled at one end with [α-^{32}P]-dATP. The products were analyzed on a neutral polyacrylamide gel.

In the absence of drug, the wild-type cleavage pattern could only be visualized by using approximate molar enzyme dimer-to-DNA ratios of 100 or 1000 (Figure 5, lanes 4 and 5; 1000 and 10000 ng, respectively). With the G269V enzyme, the cleavage pattern could be readily visualized at a molar ratio of approximately 10 (Figure 5, lane 8; 100 ng). The drug-independent cleavage pattern of the G269V protein did not differ significantly from that of the wild-type enzyme (Figure 5, lanes 5 and 8).

To assess the cleavage patterns in the presence of drug, we used an approximate molar enzyme dimer-to-DNA ratio of 1 for both enzymes (Figure 5, lanes 9−20; 10 ng), except that oxolinic acid was also tested at an approximate molar ratio of 10 in order to visualize DNA cleavage pattern of the wild-type enzyme (Figure 5, lanes 23 and 24; 100 ng). Since the observed DNA cleavage patterns are strongly influenced by the total extent of DNA cleavage, we chose inhibitor concentrations that gave us maximal levels of cleavage (based on Figures 3 and 4). As expected, each of the inhibitors altered the cleavage site specificity of both the wild-type and G269V enzymes. More importantly, the drug-dependent cleavage patterns of the mutant and wild-type proteins did not significantly differ from one another. These results indicate that the G269V mutation alters the sensitivity of the enzyme without changing the drug binding pocket, which is thought to be in intimate contact with the DNA.

DISCUSSION

The isolation and analysis of many drug-resistant mutants from bacterial, T4, and eukaryotic systems have provided a window that helps us understand the mechanism of action of topoisomerase inhibitors. In this report, we have described a new class of type II topoisomerase mutant, one that is hypersensitive to many different inhibitors that stabilize the cleavage complex. Such hypersensitive mutants should provide a different window, revealing unique aspects of the mechanism of drug action.

To facilitate this discussion, we will use the published crystal structures of the yeast type II topoisomerase (*39*, *40*) and the bacterial gyrase subunit A (*41*) as a framework for discussing G269V and other topoisomerase mutants. This approach is somewhat speculative as the structure of the T4 enzyme could differ significantly from the yeast and bacterial enzymes. However, all type IIA topoisomerases share extensive sequence homologies (*42*, *43*). Furthermore, although they represent two different enzyme conformations, the published structures of the yeast and bacterial enzymes are quite similar. Therefore, it is likely that the T4 enzyme has the same generally conserved structure. In particular, a comparison of the predicted secondary structures in the region of the G269 residue supports the validity of a conserved structure (Figure 6B).

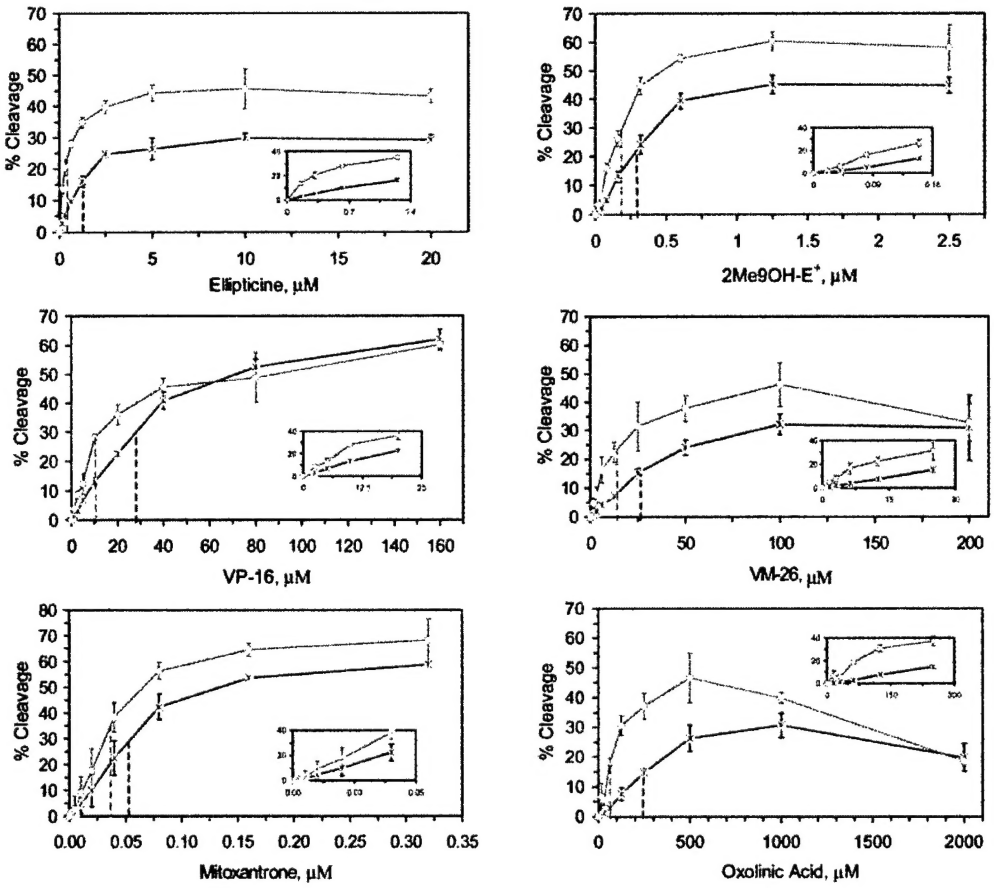

FIGURE 4: Quantitation of DNA cleavage in the presence of six different inhibitors. Each of the six drugs was tested as described in the legend to Figure 3. Key: (O) G269V; (×) wild type.

The crystal structure of a large fragment of the yeast type II topoisomerase homodimer is shown in Figure 6A (*39*). For reference, the A′ portion of the molecule is homologous to *E. coli* GyrA and the product of T4 gene *52*, while the B′ fragment is homologous to a portion of *E. coli* GyrB and the products of T4 genes *39* and *60*. Two domains of interest are the CAP-like domain and the Rossmann fold (Figure 6A; see also ref *44*). These two domains contain several charged residues that are essential for the topoisomerase cleavage reaction (*44*, *45*). All the available structural and mutational data suggest that these two regions come together to participate in catalysis and to form a drug binding pocket (*40*, *44*−*48*). Not surprisingly, the vast majority of drug resistance mutations fall into one of these two regions. For example, the two quinolone resistance determining regions (QRDR) are within the Rossmann fold and the CAP-like domain (*46*, *49*). Many of these mutations apparently alter the drug binding pocket of the enzyme because they cause differential effects on the various inhibitors (*17*, *23*−*26*, *38*).

One of the unique characteristics of the G269V substitution is its unusual location outside of the CAP-like and Rossmann fold regions. Sequence comparisons place the G269V mutation in the tower domain of the protein, between β-strands 12 and 13 (Figure 6B). On the basis of this structural alignment, residue G269 of T4 corresponds roughly to D939 and D297 of the yeast topoisomerase II and bacterial GyrA proteins, respectively. These residues are over 25 Å away from the active site tyrosine in each of the published structures. Further, the loop between β-strands 12 and 13 is located on the opposite side of the enzyme from where DNA binding is thought to occur. It therefore seems unlikely that this region of the tower domain participates directly in the formation of the drug binding pocket. Accordingly, we did not observe a change in the cleavage site specificity of the G269V mutant. One simple model is that the G269V substitution causes drug hypersensitivity simply by increasing the concentration of cleavage complexes available to drug, that is, by shifting the equilibrium of the topoisomerase reaction cycle to favor the intermediate that drug can bind. At a molar enzyme-to-DNA ratio of 1, we find that about 10% of the G269V topoisomerase dimers are participating in cleavage complexes, much higher than with the wild-type enzyme.

Three different mechanisms could conceivably lead to higher levels of cleavage complex. First, the G269V enzyme might have a higher DNA binding affinity than wild type, although this would presumably lead to a higher specific activity, which we did not observe. Second, once the enzyme is bound to the substrate, it could have a faster forward rate of cleavage. Finally, the enzyme might have a reduced rate of religation. Regardless of the specific mechanism, the effective result is an increase in the concentration of cleavage complexes available to inhibitors. In a sense, the G269V substitution works by mimicking the activity of a cleavage-enhancing inhibitor while at the same time acting synergistically with the inhibitors. Following this logic, we speculate

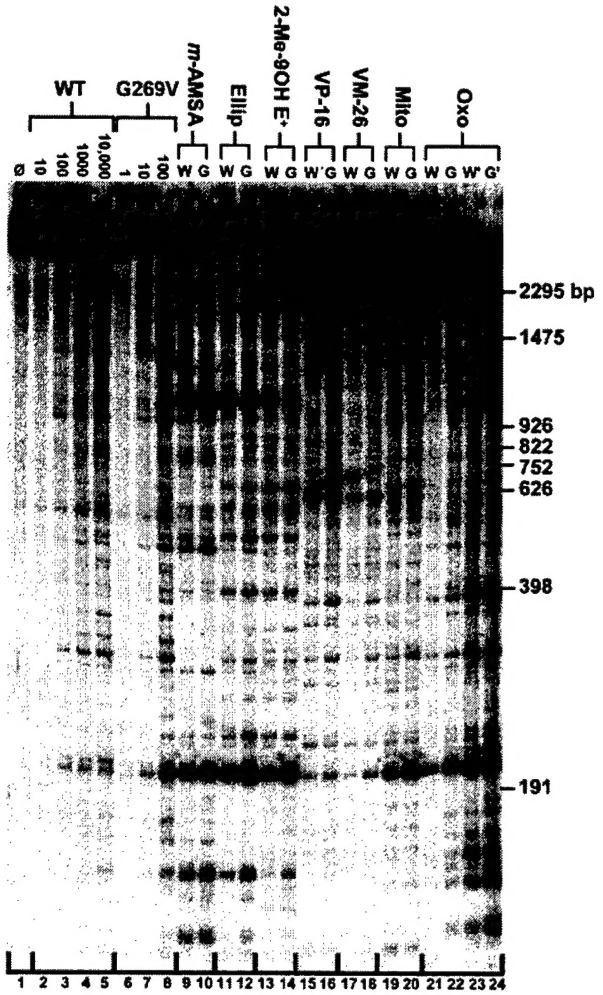

FIGURE 5: DNA cleavage patterns in the presence of different inhibitors. Reactions contained a small amount (~2 ng) of end-labeled pBR322, 100 ng of unlabeled linear pBR322 DNA (35 fmol), and the following amounts of topoisomerase: none (lane 1); increasing amounts (nanograms) indicated at top of gel (lanes 2–8); 10 ng (38.5 fmol, lanes 9–22); 100 ng (385 fmol, lanes 23 and 24). The inhibitor concentrations were as follows: *m*-AMSA, 4 μM; ellipticine, 40 μM; 2-Me-9-OH-E$^+$, 2.5 μM; VP-16, 160 μM; VM-26, 200 μM; mitoxantrone, 0.32 μM; oxolinic acid, 500 μM. The intact DNA substrate and topoisomerase-mediated cleavage products were resolved by 6% PAGE and visualized with a phosphorimager (Molecular Dynamics). A size scale (bp, base pairs), generated from the migration of pBR322 restriction fragments, appears in the right-hand margin. Key: ϕ, no enzyme and no drug; W, 10 ng of wild-type enzyme; G, 10 ng of G269V enzyme; W′, 100 ng of wild-type enzyme; G′, 100 ng of G269V enzyme.

that there might be novel drugs that mimic the activity of the G269V substitution (i.e., drugs that increase DNA cleavage without altering the cleavage site specificity of the enzyme and act synergistically with the classical cleavage-enhancing drugs).

The G269V enzyme responded to all of the drugs in a similar manner, suggesting that these drugs share a similar mechanism of action. If our interpretation of the cause of hypersensitivity is correct, then these drugs must all act by inhibiting the religation step of the enzyme. If any acted by stimulating the forward rate of cleavage, as suggested for a subset of the drugs acting on eukaryotic type II topoisomerases (*50*), hypersensitivity should not result.

The only other type II topoisomerase reported to have such high DNA cleavage levels is the wild-type enzyme of the *Chlorella* virus PBCV-1, with about 30% of its dimers in a cleavage complex at any given time (*51*). However, unlike G269V, this protein was found to be generally resistant to a number of topoisomerase poisons. A peculiarity of the *Chlorella* PBCV-1 enzyme is that even with such high levels of DNA cleavage, there did not appear to be any multiple cleavage events, suggesting that the enzyme may be highly site specific. Since drug binding sites are composed of both topoisomerase and DNA, a site-specific topoisomerase would perhaps create a drug binding pocket that is specific for a very limited number of inhibitors.

The only other reported mutant in the tower domain that affects drug sensitivity is *top2-5* from *Saccharomyces cerevisiae* (*52*). This temperature-sensitive mutant contains a cluster of three substitutions (R884P, R886I, and M887I) in β-strand 9 of the tower domain (Figure 6B). Unlike G269V, the *top2-5* mutant was shown to be resistant to *m*-AMSA and VP-16. However, similar to G269V, the cleavage site specificity of the *top2-5* mutant was not altered (in the presence of these two drugs). Although the authors did not comment on it, the *top2-5* mutant apparently yielded lower levels of drug-independent DNA cleavage (see Figures 5 and 6 in ref *52*), suggesting that this mutant might cause resistance by reducing the number of cleavage complexes available to drug.

Although other hypersensitive topoisomerase mutants have been reported, none were shown to display increased sensitivity to all inhibitors, like the G269V mutant. As described above, many other mutations cause hypersensitivity to certain drugs but resistance to others. Most of these mutations lie in the CAP-like domain or the Rossmann fold and likely alter the enzyme portion of the drug binding site. One other mutation, H1012Y of yeast topoisomerase II, is hypersensitive to ellipticine, equally sensitive to *m*-AMSA, and resistant to VP-16 and CP-115,953 (*53*), but residue 1012 lies well outside the CAP-like and Rossmann fold domains in the C-terminal portion close to the dimer interface (purple domain in Figure 6A).

Another yeast topoisomerase II mutant, T744P, showed increased sensitivity to *m*-AMSA, mitoxantrone, and some quinolones and fluoroquinolones but not to VP-16 (*54*; the ellipticines and anthracyclines, which also target the yeast enzyme, were not tested). Yeast residue T744 resides in the CAP-like domain in the region where many drug resistance mutations have been isolated (*47*). The authors suggested that the altered drug sensitivity of T744P results from a change in the cleavage/religation equilibrium (*54*). However, the mutation did not cause increased levels of drug-independent DNA cleavage as would be expected for such a change, and so the interpretation of this mutant remains uncertain.

Two human topoisomerase IIα substitutions, R450Q and G437E, have been reported to show a correlation between ATPase function and drug sensitivity (*55*). Both of these substitutions are located near the N-terminus of the Rossmann fold adjacent to the ATPase domain of the protein. The R450Q mutant enzyme displayed a lower ATPase activity and was found to be resistant to *m*-AMSA, doxorubicin, mitoxantrone, and VM-26 in the presence and absence of ATP (quinolones and ellipticines were not tested).

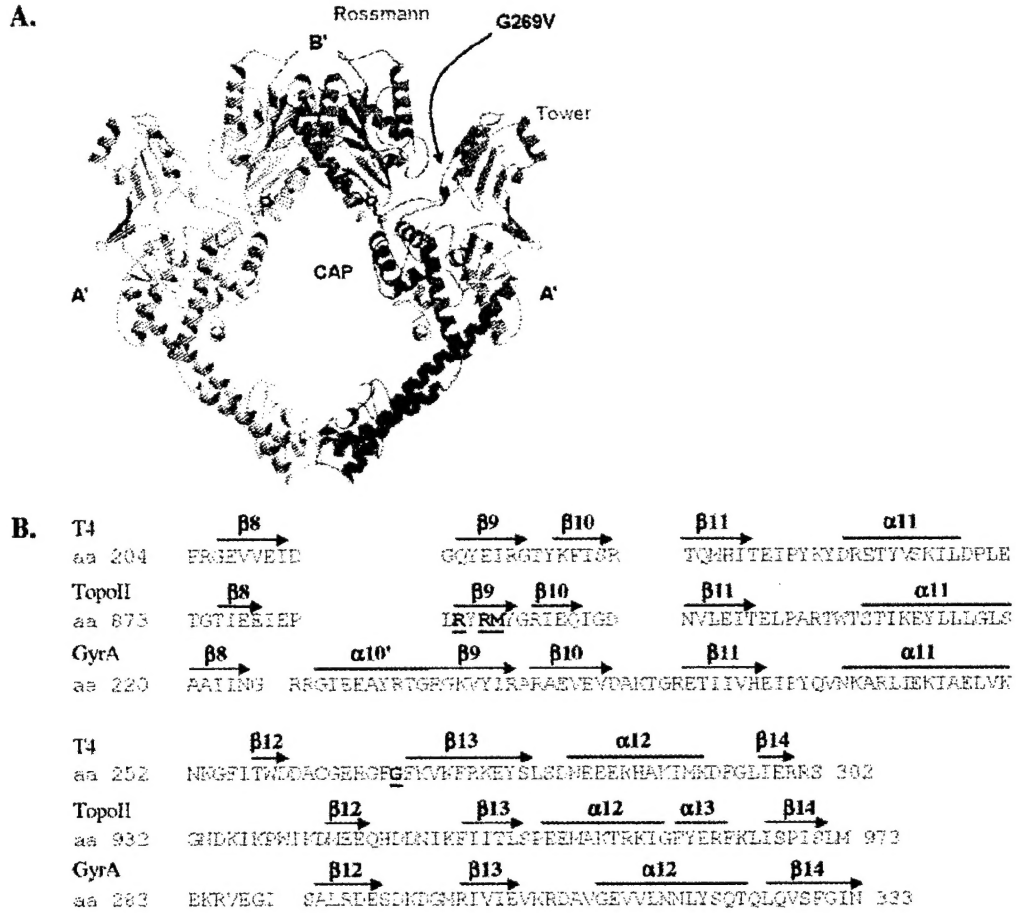

FIGURE 6: Structural context of the G269V substitution. A ribbon diagram of a large fragment of yeast topoisomerase II is shown in (A) (*39*). This figure was generated with the program RIBBONS (*57*). One protomer is colored, and the domains discussed in the text are as follows: CAP-like domain, dark blue; Rossmann fold, red; tower domain, green; and the C-terminal domain that participates in dimerization, purple. The active site tyrosine is a part of the CAP-like domain, and both tyrosines are shown in black. Secondary structure overlays of the tower domain from three topoisomerase proteins are shown in (B). The β-strands are indicated by arrows while the α-helices are shown as bars. The TopoII and GyrA alignments and structural nomenclature are based on published three-dimensional structures and alignments (*39*, *41*). The T4 gp52 structural elements were predicted using the PHDsec program (*58*, *59*). The tower domain mutants discussed in the text are underlined and in bold.

In contrast, the G437E mutant enzyme was reported to utilize ATP more efficiently and, like our mutant, display increased levels of drug-independent cleavage. Compared to wild type, the G437E mutant enzyme was equally sensitive to cleavage-inducing drugs in the presence of ATP but hypersensitive to multiple drugs in the absence of ATP. However, the G437E mutant enzyme in the absence of ATP was no more sensitive than the wild-type enzyme in the presence of ATP. In other words, the G437E enzyme was simply unaffected by the presence or absence of ATP (see Figure 7 in ref *55*). Therefore, one interpretation of these results is that the G437E substitution uncouples DNA cleavage from its normal ATP dependence. These results support a correlation between ATPase activity and DNA cleavage levels for the human enzyme and raise the possibility that the T4 wild-type or G269V mutant enzyme might show a similar coupling. However, we find that the G269V mutant enzyme is equally hypersensitive to *m*-AMSA and oxolinic acid in both the absence and presence of ATP and that DNA cleavage by the wild-type T4 enzyme is also ATP independent (E. O'Reilly and K. Kreuzer, unpublished data; also see ref *56*).

The G269V substitution was originally isolated in combination with S79F in a selection for drug-resistant mutants (*22*, *31*). S79F by itself causes a topoisomerase negative phenotype in vivo, which is rescued by the G269V substitution. Comparisons with the yeast enzyme place the S79 residue in the CAP-like domain of the protein, far from the G269V substitution in the tower domain. What is the nature of the defect of the S79F mutant and how does G269V suppress this defect? Also, why is the drug resistance phenotype dominant to the hypersensitivity phenotype in the double mutant? We are currently trying to answer these questions.

In summary, we have described a T4 topoisomerase mutant that is hypersensitive to multiple classes of type II topoisomerase poisons. This mutation is located in a region of the protein that has not been previously implicated in drug or DNA binding. The G269V substitution increases the number of cleavage complexes available to inhibitors, apparently by altering the equilibrium of the topoisomerase reaction cycle. We believe this is the first direct demonstration that a type II topoisomerase mutation can alter drug

sensitivity by altering the equilibrium of the enzyme. Analysis of this and similar mutants provides a unique perspective on the mechanism of action of topoisomerase inhibitors.

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